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REVIEW

Covalent control of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: Insights into autoregulation of a bifunctional enzyme

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Abstract

The hepatic bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2-K/Fru-2,6-P_2ase), E.C. 2-7-1-105/E.C. 3-1-3-46, is one member of a family of unique bifunctional proteins that catalyze the synthesis and degradation of the regulatory metabolite fructose-2,6-bisphosphate (Fru-2,6-P_2). Fru-2,6-P_2 is a potent activator of the glycolytic enzyme 6-phosphofructo-1-kinase and an inhibitor of the gluconeogenic enzyme fructose-1,6-bisphosphatase, and provides a switching mechanism between these two opposing pathways of hepatic carbohydrate metabolism. The activities of the hepatic 6PF-2-K/Fru-2,6-P_2ase isoform are reciprocally regulated by a cyclic AMP-dependent protein kinase (cAPK)-catalyzed phosphorylation at a single NH_2-terminal residue, Ser-32. Phosphorylation at Ser-32 inhibits the kinase and activates the bisphosphatase, in part through an electrostatic mechanism. Substitution of Asp for Ser-32 mimics the effects of cAPK-catalyzed phosphorylation. In the dephosphorylated homodimer, the NH_2- and COOH-terminal tail regions also have an interaction with their respective active sites on the same subunit to produce an autoregulatory inhibition of the bisphosphatase and activation of the kinase. In support of this hypothesis, deletion of either the NH_2- or COOH-terminal tail region, or both regions, leads to a disruption of these interactions with a maximal activation of the bisphosphatase. Inhibition of the kinase is observed with the NH_2-truncated forms, in which there is also a diminution of cAPK phosphorylation to decrease the K_m for Fru-6-P. Phosphorylation of the bifunctional enzyme by cAPK disrupts these autoregulatory interactions, resulting in inhibition of the kinase and activation of the bisphosphatase. Therefore, effects of cyclic AMP-dependent phosphorylation are mediated by a combination of electrostatic and autoregulatory control mechanisms.

Keywords: bifunctional enzymes; cyclic AMP-dependent phosphorylation; fructose-1,6-bisphosphatase inhibitor; hepatic carbohydrate metabolism; 6-phosphofructo-1-kinase inhibitor; 6 phosphofructo-2-kinase/fructose-2,6-bisphosphatase

All processes in biology are regulated, and the hormonal regulation of hepatic glucose production and utilization is brought about by the covalent modification of several key regulatory enzymes in the pathway, as well as by transcriptional control (reviewed in Pilkis & Claus, 1991). In general, phosphorylation is the most common covalent modification of proteins, others being ADP-ribosylation, methylation, and acetylation. These control mechanisms regulate cellular activity in balance with concurrent actions of enzymes, which can reverse these covalent modifications. Phosphorylation/dephosphorylation of hydroxyl groups on amino acid side chains is the predominant acute regulatory control mechanism for hepatic glucose metabolism. In two cases, muscle glycogen phosphorylase (reviewed in Johnson, 1992, 1994), and isocitrate dehydrogenase (Dean & Koshland, 1990; Hurley et al., 1990), X-ray crystallographic analysis has revealed two different mechanisms, allosteric and electrostatic, respectively, for mediating covalent control of proteins by phosphorylation. Muscle glycogen phosphorylase is regulated by allosteric effects between the phospho-Ser-14 and the catalytic site, which are separated by approximately 30 Å (reviewed in Johnson, 1992, 1994). Phosphorylation at Ser-14 results in concerted tertiary and quaternary structural changes

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resulting in the catalytic residues moving into an active conformation, and the opening of access for substrates. Isocitrate dehydrogenase is regulated directly by the electrostatic effects induced by phospho-Ser-113 at the active site, which prevent the negatively charged citrate substrate from binding (Dean & Koshland, 1990).

In addition to covalent modification, autoregulation is another cellular control mechanism for key regulatory proteins. Many protein kinases are self-regulated by an intrasteric mechanism where part of the enzyme's structure acts as a pseudosubstrate and directly inhibits catalysis at the active site (reviewed in Soderling, 1990; Kemp & Pearson, 1991; Kemp et al., 1994). The term intrasteric regulation emphasizes regulation directed at the active site. This is in contrast to allosteric regulation, where the ligand or a part of the enzyme that has been covalently modified, such as phospho-Ser-14 of muscle glycogen phosphorylase, acts at an alternative site. Two recently determined X-ray crystallographic protein structures, twitchin kinase (*Caenorhabditis elegans* fragment residues 5890–6262), and cyclic AMP-dependent protein kinase (cAPK) are bilobal structures shown to have similar autoregulatory intrasteric mechanisms (Hu et al., 1994). For twitchin kinase, its COOH-terminal tail acts as a pseudosubstrate, binding in a cleft between the two lobes that contain a hinge with adjacent glycine residues in proximity to an ATP-binding glycine-rich loop, making extensive autoinhibitory contacts with the catalytic core. Removal of this pseudosubstrate sequence from members of the twitchin/myosin light chain kinase family activates them (reviewed in Kemp & Pearson, 1991; Kemp et al., 1994). Allosteric control is required to modulate intrasteric interactions (reviewed in Kemp & Pearson, 1991). For twitchin kinase and myosin light chain kinase, relief of this intrasteric inhibition is thought to occur through binding calcium/calmodulin to recognition sequences in the COOH-terminal tail (Knighton et al., 1992; Heierhorst et al., 1994). cAPK is activated by the cAMP-induced dissociation of cAMP-binding regulatory subunits from the catalytic subunits. The regulatory subunit contains a pseudosubstrate region that shares a target-like sequence for the kinase (reviewed in Taylor et al., 1993). This process converts inactive, regulatory subunit–catalytic subunit complexes to active, free catalytic subunits. The X-ray crystallographic structure of cAPK reveals the binding of a 20-amino acid inhibitory peptide to the active site of the kinase. Binding of the substrate-like peptide induces a closing around the protein kinase active site, which lies at the base of the cleft between the smaller nucleotide binding lobe and the larger target peptide-recognition lobe (Zheng et al., 1993).

Our recent studies on the covalent regulation of the bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-P_2ase (6PF-2-K/Fru-2,6-P_2ase), suggest that its regulation by cAPK-catalyzed phosphorylation is mediated by a combination of electrostatic, allosteric, and autoregulatory control mechanisms that may also involve an intrasteric mechanism. Regulation of the bifunctional enzyme by cAPK is crucial to hepatic metabolic signaling, and provides a switching mechanism between glycolysis and gluconeogenesis in mammalian liver (reviewed in Pilkis & Claus, 1991). The simultaneous operation of fructose-1,6-bisphosphatase (Fru-1,6-P_2ase) and 6-phosphofructo-1-kinase (6PF-1-K) in the glycolytic/gluconeogenic pathway (Fig. 1) represents how the rate and direction of flux are regulated by small changes in the concentration of an allosteric effector, fructose-2,6-bisphosphate (Fru-2,6-P_2) in this case. 6PF-2-K/Fru-2,6-P_2ase catalyzes both the synthesis and degradation of Fru-2,6-P_2, which is an activator of 6PF-1-K and an inhibitor of Fru-1,6-P_2ase. The rat liver bifunctional enzyme is a homodimer whose activities are regulated by cAPK-catalyzed phosphorylation at a single NH_2-terminal seryl residue (Ser-32), whose phosphorylation results in the activation of the Fru-2,6-P_2ase and inhibition of the 6PF-2-K (Murray et al., 1984). This review will summarize the present understanding of how phosphorylation regulates the bifunctional enzyme, resulting in the control of hepatic glucose production and utilization.

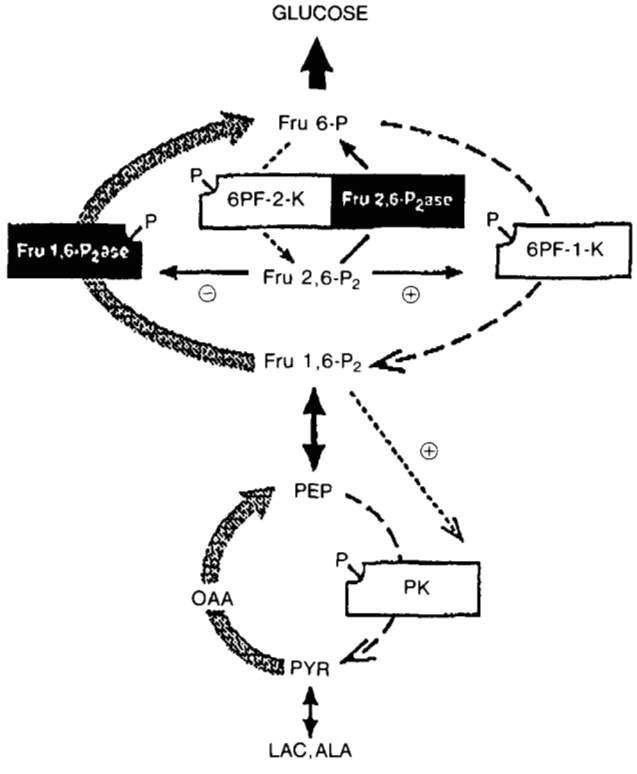

Fig. 1. Sites of short-term regulation of hepatic gluconeogenesis by cAMP. Starvation results in an elevation of liver cAMP levels, which leads to phosphorylation of pyruvate kinase (PK), 6PF-1-K, Fru-1,6-P_2ase, and 6PF-2-K/Fru-2,6-P_2ase, indicated by -P attached to the appropriate enzyme box. Unlike PK or 6PF-2-K/Fru-2,6-P_2ase, phosphorylation of 6PF-1-K and Fru-1,6-P_2ase does not result in a change that is kinetically significant. 6PF-1-K is activated and Fru-1,6-P_2ase is inhibited by Fru-2,6-P_2, the level of which is decreased when 6PF-2-K/Fru-2,6-P_2ase is phosphorylated. Decreases in Fru-2,6-P_2 result in a lower Fru-1,6-P_2 level, which contributes to the inactivation of PK both allosterically and by making PK a better substrate for cAPK. Solid boxes, enhanced enzyme activity; open boxes, enzyme inhibition. Shaded arrows, increased flux; dashed arrows, diminished flux. ALA, alanine; LAC, lactate.

Isoforms of 6PF-2-K/Fru-2,6-P_2ase

Since the discovery of rat liver 6PF-2-K/Fru-2,6-P_2ase (El-Maghrabi et al., 1982a, 1982b; Lively et al., 1988), four other mammalian isoforms have been identified in skeletal muscle (Darville et al., 1989; Kitamura et al., 1989), heart (Kitamura & Uyeda, 1987; Tsuchya & Uyeda, 1994), testis (Sakata et al., 1991), and brain (Ventura et al., 1992). The liver and skeletal

muscle isoforms are alternative splicing products of a single gene (Darville et al., 1989), and the heart, testis, and brain isoforms are each products of other distinct genes. In all of the isoforms, there is a high degree of conservation of the core structure of both the kinase and bisphosphatase domains (Fig. 2). The major difference between the members of this bifunctional enzyme family is the length and composition of the NH_2- and/or COOH-terminal regions, and the presence or absence of various protein kinase phosphorylation sites in these regions. For example, bovine heart muscle 6PF-2-K/Fru-2,6-P_2ase exists in two forms that are generated by alternative splicing, a 58-kDa form and a 54-kDa form (Rider et al., 1992a). Although the primary sequence of the 58-kDa heart form and the liver form core domains are 86% identical, the NH_2- and COOH-terminal regions are only 29% and 42% identical, respectively (Lange et al., 1991). The rat liver isoform has a 32-amino acid NH_2-terminus that contains a single cAMP-dependent protein kinase site at Ser-32 (Murray et al., 1984), whereas the 58-kDa bovine heart isoform has a 30-amino acid NH_2-terminus with a corresponding consensus cAMP phosphorylation site at Ser-29 (Kitamura et al., 1988; Lange et al., 1991). This bovine heart form also contains two additional phosphorylation sites in the COOH-terminal region, Ser-466 and Thr-475 (Fig. 2), which extends 60 amino acids beyond that of the liver isoform. The Ser-466 site is a cAMP-dependent protein kinase site, whereas Thr-475 is a protein kinase C site (Kitamura et al., 1988; Rider et al., 1992b). A second possible protein kinase C phosphorylation site may be at Ser-84 (Rider et al., 1992b).

The brain isoform has extended NH_2- and COOH-terminal regions, such that the enzyme is twice the size (110 kDa) of that of the liver form (55 kDa) (Ventura et al., 1992, 1995). It also has a consensus cAPK site just N-terminal to the core kinase domain. Neither the muscle nor the testis isoforms contain documented phosphorylation sites. The testis isoform is similar in size to the liver enzyme (Sakata et al., 1991), and does not contain a protein kinase phosphorylation site (Abe & Uyeda, 1994). The muscle isoform, which does not contain a cAMP-dependent protein–kinase phosphorylation site, has 9 unique NH_2-terminal amino acids in place of the 32 amino acids of the liver form (Crepin et al., 1989; Darville et al., 1989).

Two yeast forms of 6PF-2-K/Fru-2,6-P_2ase have also been identified (Fig. 2). Yeast PFK26 is similar in size to the brain isoform, with extra coding sequences at both termini (Kretschmer & Fraenkel, 1991). It contains a cAMP-dependent protein kinase phosphorylation site just COOH-terminal to the core bisphosphatase domain (Kretschmer & Fraenkel, 1991). The other yeast isoform, FBP26, is similar to the skeletal muscle isoform, both in size and in lack of phosphorylation sites (Paravicini & Kretschmer, 1992).

In addition to having a core structure that is highly conserved across tissues, there is a high degree of identity of tissue-specific 6PF-2-K/Fru-2,6-P_2ase isoforms across different species. For

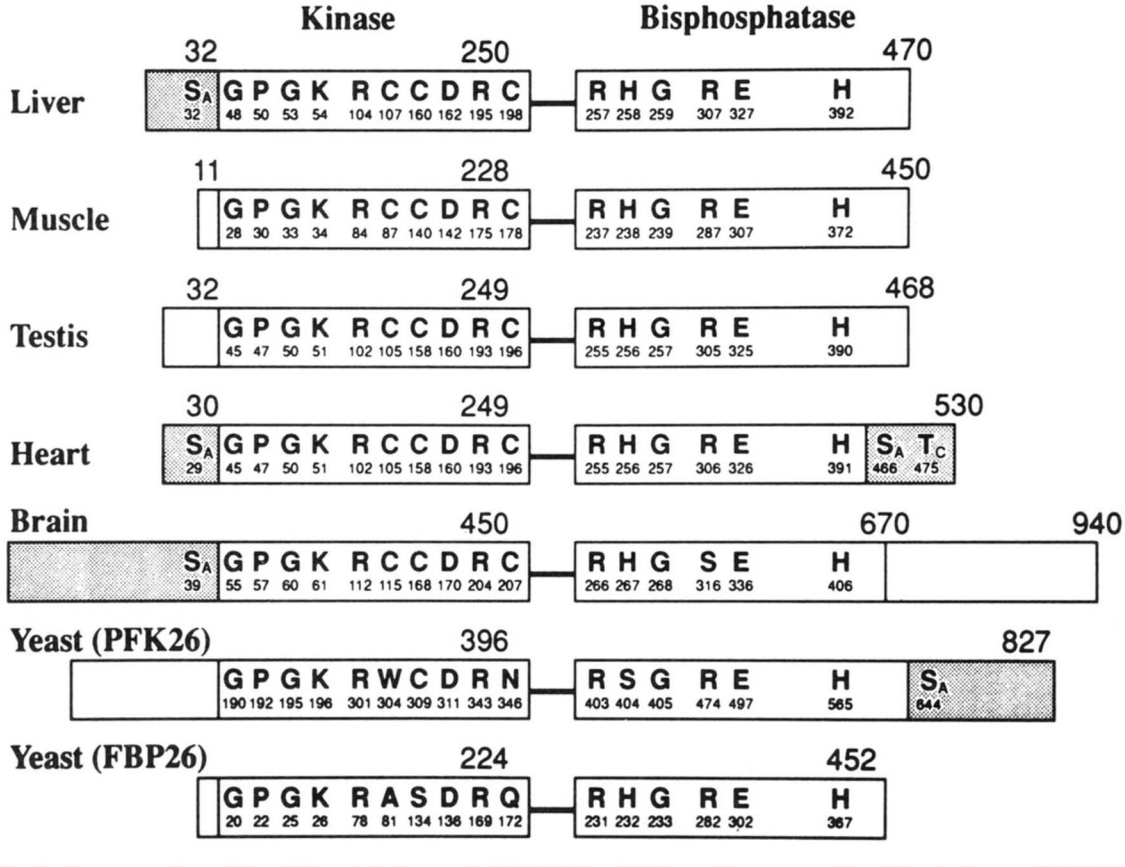

Fig. 2. Representation of the different isoforms of 6PF-2-K/Fru-2,6-P_2ase. There are at least five isoforms of 6PF-2-K/Fru-2,6-P_2ase in mammalian tissues: a liver form, a muscle form, a heart form, a testis form, and a brain form. In addition, there are two known isoforms of 6PF-2-K/Fru-2,6-P_2ase in yeast. Regions that regulate the enzyme after being phosphorylated are shaded, and are typically at the NH_2- and/or COOH-termini of all forms.

example, the identity at the amino acid level of the human (Lange & Pilkis, 1990; Lange et al., 1993) and rat (Colosia et al., 1987; Darville et al., 1987) liver enzymes to the bovine liver (Lange et al., 1991) enzyme is 98.3% and 97%, respectively. The chicken liver enzyme has 89.1%, 88.4%, and 88.6% identity to the human, rat, and bovine enzymes, respectively (Li et al., 1993).

Even though their core structures are highly conserved, there are differences in kinase and bisphosphatase properties between the tissue-specific isoforms. As shown in Table 1, the rat liver, bovine testis, and bovine brain isoforms have similar V_{max} values for their kinases and bisphosphatases, and thus have similar kinase to bisphosphatase ratios. However, the skeletal muscle isoform has a low kinase to bisphosphatase activity ratio, whereas the bovine heart enzyme has a high kinase to bisphosphatase activity ratio. Other significant differences between these isoforms are the affinity of the skeletal muscle kinase for Fru-6-P, which is 40-fold lower than that for the liver isoform, and the affinity of the bisphosphatase for Fru-2,6-P_2, which is at least 100–1,000-fold greater for the liver and skeletal muscle isoforms than for the testis, brain, and heart forms. Because the NH_2- and COOH-termini are the areas of least homology for these tissue-specific isoforms, it seems reasonable to assume that these regions are responsible for their kinetic differences, both in the presence and absence of phosphorylation. Table 1 also shows the kinetic properties of a "catalytic core" bifunctional enzyme, in which the first 22 NH_2-terminal and last 30 COOH-terminal amino acids were deleted from the liver enzyme, leaving intact the kinase and bisphosphatase domains (Kurland et al., 1995a). This form has a low kinase to bisphosphatase activity ratio. The yeast isoform FBP26 (Paravicini & Kretschmer, 1992), and the skeletal muscle isoform (Crepin et al., 1992; Kurland et al., 1993a) also have low kinase to bisphosphatase activity ratios. The "catalytic core" form and the skeletal muscle enzyme not only have very low kinase to bisphosphatase ratios, but their kinase activities are also similar, and very low, relative to that of the liver enzyme (Kurland et al., 1995b). At the other extreme is the bovine heart enzyme, which has a high kinase to bisphosphatase ratio, supporting the hypothesis that the NH_2- and COOH-termini of the various bifunctional enzyme isoforms serve to adapt the kinetic properties of the catalytic core to the metabolic exigencies of a particular tissue (Kurland et al., 1993b).

Structure/function relationships of 6PF-2-K/Fru-2,6-P_2ase

The bifunctional enzyme has been hypothesized to arise via a gene fusion event from a primordial bacterial 6-phosphofructo-1-kinase and a primordial mutase/phosphatase (Bazan et al., 1989). A most distinctive characteristic of both activities of the bifunctional enzyme is their very low turnover numbers of 6 min^{-1}, which are two to three orders of magnitude lower than that for most phosphotransferases or phosphohydrolases.

6-Phosphofructo-2-kinase

The kinase prefers ATP as a phosphate donor, but can also utilize GTP (El-Maghrabi et al., 1981). The preferred phosphate group acceptor is D-Fru-6-P, although L-sorbose-6-phosphate can also serve as an acceptor (Pilkis et al., 1985). The pH optimum is between 8 and 9 (El-Maghrabi et al., 1984b; Van Schaftingen et al., 1981). The reaction is not inhibited by ATP (El-Maghrabi et al., 1981), in contrast to mammalian 6-PF-1-Ks. Inorganic phosphate increases the affinity of 6PF-2-K for Fru-6-P under physiologic conditions without affecting the K_m for ATP (Van Schaftingen et al., 1981; Kountz et al., 1986), as does arsenate (Kountz et al., 1986). Citrate, phosphoenolpyruvate (Van Schaftingen et al., 1981), and glycerol-3-phosphate (Claus et al., 1982) are physiological inhibitors of 6PF-2-K, and AMP may be a possible activator (Van Schaftingen et al., 1981).

The kinase reaction is also inhibited by both of its products, ADP and Fru-2,6-P_2 (Kitajima et al., 1984). The former is

Table 1. *Comparison of the kinetic properties of the kinase and bisphosphatase between the tissue-specific isoforms of 6PF-2-K/Fru-2,6-P_2ase and the catalytic core of the liver form*[a]

	Liver	Liver, catalytic core	Skeletal muscle, rat	Heart, bovine	Testes, bovine	Brain, bovine
Size: no. amino acids	470	418	450	570	468	940
Kinase						
V_{max} (mU/mg)	113	16	5.3	180	90	90
K_m Fru-6-P (μM)	20	753	803	50	16	27
V_{max}/K_m	5.6	0.02	0.007	3.6	5	3.3
Bisphosphatase						
V_{max} (mU/mg)	45	194	112	2.2	22	29
K_m Fru-2,6-P_2 (μM)	0.005	0.01	0.01	40	>1	0.4
V_{max}/K_m	9,000	19,400	11,200	0.055	22	0.4
E-P formation (mol/mol)	1.0	1.0	1.0	0.05	0.6	0.7
V_{max} ratio: kinase/bisphosphatase	2.5	0.08	0.05	80	4.0	3.0

[a] Data for the native and catalytic core liver and skeletal muscle isoforms are from the authors' laboratory (Kurland et al., 1995). Data for the heart (El-Maghrabi et al., 1986; Sakata & Uyeda, 1990; Sakata et al., 1991), brain (Ventura et al., 1992), and testis (Sakata et al., 1991) isoforms are from the literature. Units for bisphosphatase E-P formation (mol/mol) are mole of ^{32}P incorporated from Fru-2-^{32}P,6-P per mole of enzyme subunit.

competitive with ATP, whereas the latter is noncompetitive with either substrate. This pattern is consistent with a sequential ordered mechanism where ATP binds first, followed by Fru 6-P. Analysis of the stereochemical course of the reaction was consistent with the transfer of the phospho group between the two substrates without a phosphoenzyme intermediate (Kountz et al., 1988), and no intermediate has been detected.

Mammalian 6PF-2-K is but one member of a family of phosphofructokinases (Bazan et al., 1989). Phosphofructokinases from bacteria, yeast, plants, and mammals have analogous structures that have resulted from gene duplication of a bacterial catalytic unit. Sequence comparisons of 6PF-2-K with 6PF-1-Ks indicate that a similar nucleotide binding fold is present in all ATP-dependent 6PF-1-Ks as well as in the 6PF-2-K domain of the bifunctional enzyme, suggesting that the structure of the phosphofructokinase active site in all these enzymes has been conserved (Bazan et al., 1989). Figure 3A shows a partial sequence alignment of rat liver 6PF-2-K with bacterial 6PF-1-K, in which structural and substrate binding residues predicted to be common and important to both 6PF-1-K and 6PF-2-K are given, and Figure 3B shows a partial topology diagram of rat liver 6PF-2-K that is based upon the features of this alignment. The proposed alternating α-helix/open β-sheet structure of 6PF-2K consists of a large and a small subdomain. The active site is proposed to lie in a cleft between these two subdomains, which in general is where active sites are found when two α-helix/open β-sheet domains face each other (Branden & Tooze, 1991).

Site-directed mutagenesis of residues within the Gly_{48}-X-Pro_{50}-X_2-Gly_{53}-Lys_{54} 6-PF2-K sequence (Fig. 3A) confirmed that it was a nucleotide binding fold (nbf) signature sequence. For example, mutation of Gly-48 to Ala in the rat liver enzyme completely eliminated kinase activity without affecting bisphosphatase activity (Li et al., 1992b), and the same results were obtained when Pro-50 or Gly-53 were mutated to Ala, or when Lys-54 was mutated to Gln (Kurland et al., 1995b). Another highly conserved sequence in 6PF-2-K, $R_{195}IEC_{198}YEI$, matched the *Bacillus stearothermophilus* $R_{162}IYV_{165}IEV$ residues (Fig. 3A). The latter motif is highly conserved in all 6PF-1-K sequences, and based on this homology modeling to bacterial 6PF-1-K, the arginine residues of 6PF-2-K, Arg-195, Arg-230, and Arg-238 were predicted to be involved in Fru-6-P binding (Bazan et al., 1989). Mutation of Arg-195 to Ala or His had no effect on V_{max} of the kinase but increased the K_m for Fru-6-P more than 3,000-fold and the K_a for phosphate by 100-fold (Li et al., 1992b). Mutation of the adjacent basic residue, Lys-194, to Ala had no effect on V_{max} or the K_m for Fru-6-P. Mutation of either

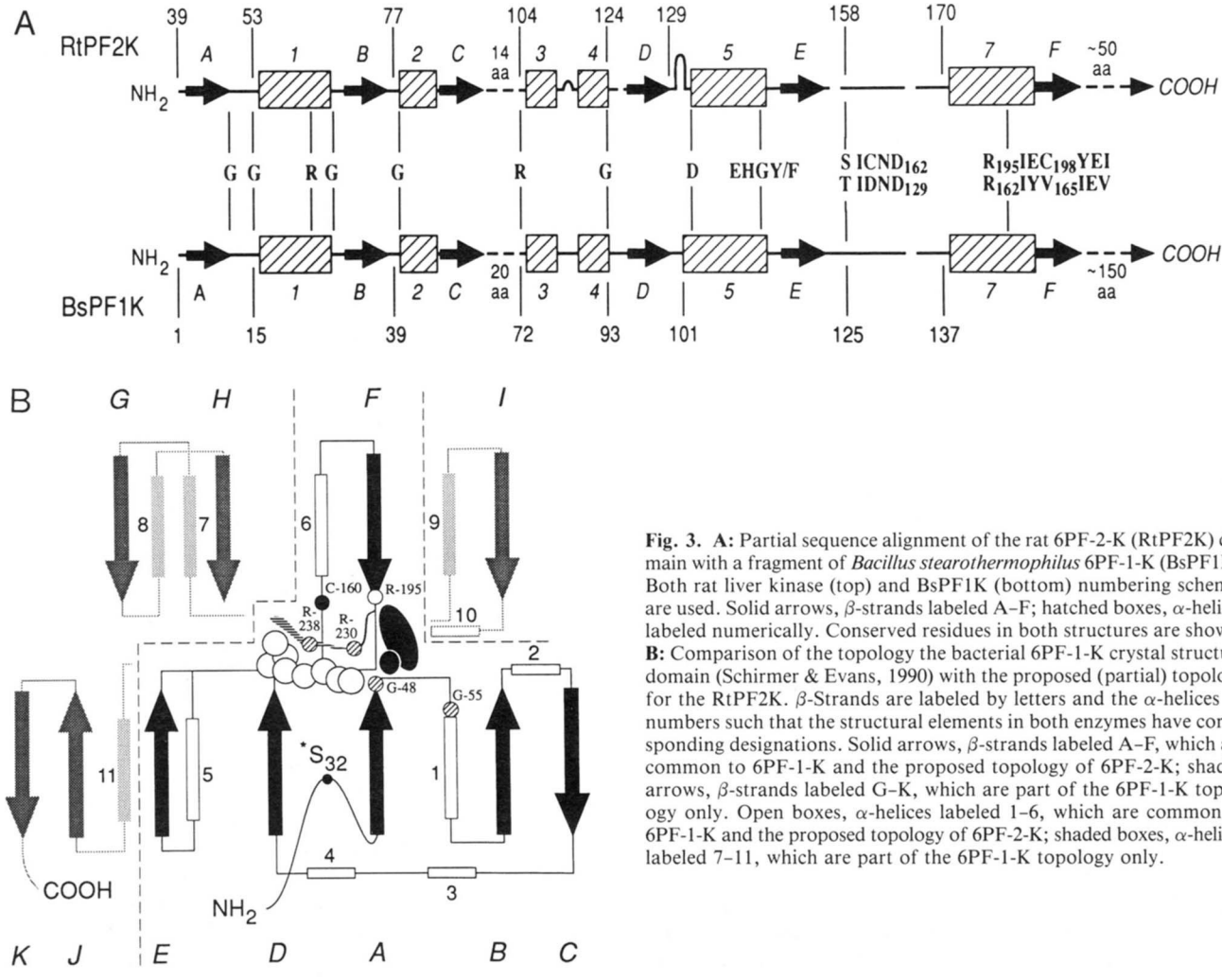

Fig. 3. A: Partial sequence alignment of the rat 6PF-2-K (RtPF2K) domain with a fragment of *Bacillus stearothermophilus* 6PF-1-K (BsPF1K). Both rat liver kinase (top) and BsPF1K (bottom) numbering schemes are used. Solid arrows, β-strands labeled A–F; hatched boxes, α-helices labeled numerically. Conserved residues in both structures are shown. **B:** Comparison of the topology the bacterial 6PF-1-K crystal structure domain (Schirmer & Evans, 1990) with the proposed (partial) topology for the RtPF2K. β-Strands are labeled by letters and the α-helices by numbers such that the structural elements in both enzymes have corresponding designations. Solid arrows, β-strands labeled A–F, which are common to 6PF-1-K and the proposed topology of 6PF-2-K; shaded arrows, β-strands labeled G–K, which are part of the 6PF-1-K topology only. Open boxes, α-helices labeled 1–6, which are common to 6PF-1-K and the proposed topology of 6PF-2-K; shaded boxes, α-helices labeled 7–11, which are part of the 6PF-1-K topology only.

Arg-230 or Arg-238 to Ala increased the K_m for Fru-6-P by two- to threefold, but, more significantly, also increased the K_m for ATP by 30–40-fold. These results indicate that Arg-195 is a critical residue for the binding of Fru-6-P, and that this interaction is highly specific because mutation of the adjacent Lys-194 to Ala had no effect on Fru-6-P binding. Arg-195 also apparently plays an important role in phosphate binding. The other two arginine residues, Arg-230 and Arg-238, which are shown to be important Fru-6-P binding residues in bacterial 6PF-1-K (Valdez et al., 1989; Berger & Evans, 1990; Schirmer & Evans, 1990), are involved principally in ATP binding in 6PF-2-K (Bazan et al., 1989).

Cys-160 is part of a highly conserved sequence (EHGY-[6AA]-SIC_{160}ND) that is part of the active site cavity of 6PF-1-K (EHGF-[7AA]-TID_{127}ND) and is therefore predicted to lie in the 6PF-2-K active site cleft (Fig. 3A,B). In the 6PF-2-K family, the Cys-160 residue is highly conserved even in the yeast 6PF-2-K forms, and site-directed mutagenesis studies indicate that Cys-160 plays an important role in the structural stability of the enzyme (Kurland et al., 1993a). Consideration of the relative specific activities of the 6PF-2-K (50 mU/mg) and 6PF-1-Ks (~100 U/mg) also support the hypothesis that the 6PF-2-K reaction may proceed without the intervention of a strong base catalyst. In bacterial 6PF-1-K, Asp-127 serves as a base catalyst in the reaction (Hellinga & Evans, 1985; Shirakihara & Evans, 1988), and mutation of the base catalytic residue Asp-127 to serine reduced this mutant's 6PF-1-K activity to a level roughly equivalent to skeletal muscle and liver 6PF-2-Ks (Hellinga & Evans, 1985). It has been hypothesized that 6PF-2-K catalysis may be mediated solely by preferential binding of the substrates in the transition state compared to the ground state, similar to the mechanism of stabilization of enzyme transition states by catalytic antibodies (Benkovic, 1992).

Fructose-2,6-bisphosphatase

The bisphosphatase specifically hydrolyzes phosphate from the C-2 position of Fru-2,6-P_2 and it does not hydrolyze Fru-1,6-P_2 or Glu-1,6-P_2, nor does it hydrolyze phosphate from the C6 position (El-Maghrabi et al., 1982b; Van Schaftingen & Hers, 1982). Catalysis of the bisphosphatase reaction involves the formation and breakdown of a 3-phosphohistidine reaction intermediate (Stewart et al., 1985), and the steady-state phosphoenzyme level correlates well with the hydrolytic rate (Stewart et al., 1985). The reaction does not require divalent cations (Van Schaftingen & Hers, 1982), has a pH optimum of 5.5–6.5 (Van Schaftingen & Hers, 1982; El-Maghrabi et al., 1984b; Stewart et al., 1985), and is inhibited by both the substrate, Fru-2,6-P_2, and the product of the reaction, Fru-6-P (Van Schaftingen & Hers, 1982; Stewart et al., 1985). The K_m for Fru-2,6-P_2 is 5 nM, and micromolar substrate concentrations inhibit the reaction (Stewart et al., 1985). Inorganic phosphate and glycerol-3-phosphate inhibit Fru-2,6-P_2ase activity at subsaturating substrate concentrations by antagonizing substrate binding and thus increase the apparent K_m 20-fold (Stewart et al., 1985). At saturating substrate concentrations, phosphate and glycerol-3-phosphate activate Fru-2,6-P_2ase by antagonizing product binding and substrate inhibition and thus increase the K_i for Fru 6-P (Stewart et al., 1985).

The Fru-2,6-P_2ase domain was modeled on the structure of yeast phosphoglycerate mutase (PGM), based on the similarity of the sequence surrounding the phosphohistidine in both enzymes (Bazan et al., 1989; Fig. 4). The yeast PGM structure is a typical three-layer α/β-fold: a largely parallel β-sheet core sandwiched by α-helices (Winn et al., 1981). The active site lies in a crevice at the COOH-terminal end of the β-sheet, a site topologically favored in structures similar to nbfs (Branden, 1980). PGM does not bind nucleotides, yet assumes an nbf-like α/β structure favorable for binding phosphoglycerates and for spatial grouping of catalytic residues far apart in sequence. In particular, His-179 is juxtaposed with the phosphorylated His-8, and the two histidine residues form a "clapping hands" structure. The alignment of Figure 4 illustrates significant similarities of the predicted secondary structural elements of Fru-2,6-P_2ase, yeast phosphoglycerate mutase (YePGM), and human prostatic acid phosphatase (HuAcP), with the greatest difference being the chain length between the active-site histidyl residues. Recently an α-ribazole-phosphate phosphatase from *Salmonella typhimurium* (O'Toole et al., 1995) was found also to belong to this family. The reactions catalyzed by the phosphatase/mutase enzyme family all involve the removal or transfer of a phosphate on a sugar backbone.

The phosphohistidine in Fru-2,6-P_2ase, His-258, is located only eight residues from the presumed NH_2-terminus of the domain (amino acid 251). The companion to His-258 is His-392 (Tauler et al., 1990). This assignment was the result of a secondary structure study that located similar α and β secondary elements in Fru-2,6-P_2ase to those found in yeast PGM, or that were predicted to occur in other homologous mutases (Bazan et al., 1989). Thus, the spacing between the two His residues is 134 amino acids rather than the 170 in yeast PGM.

The catalytic center of the rat liver Fru-2,6-P_2ase domain involves the triad His-258, Glu-327, and His-392. The conservation of the Arg-His-Gly motif in the PGM and Fru-2,6-P_2ase families (Pilkis et al., 1987), the demonstration of a functional homology of Fru-2,6-P_2ase and PGM (Tauler et al., 1987), and the observation that His-258 was labeled upon incubation with [2-^{32}P]Fru-2,6-P_2 (Stewart et al., 1985) all pointed to His-258 being the phosphoacceptor in rat liver Fru-2,6-P_2ase. The importance of His-258 was confirmed when it was mutated to Ala, and the mutant protein was found to be devoid of bisphosphatase activity and did not form a phosphoenzyme intermediate (Tauler et al., 1990). His-392 by its homology to yeast PGM (Winn et al., 1981), has been predicted to form a "clapping hands" structure, and this has been confirmed by X-ray crystal structure determination of a 30-amino acid truncated mutant of the Fru-2,6-P_2ase (Lee et al., 1995). Glu-327, which is conserved in all known Fru-2,6-P_2ases and PGM, is hypothesized to stabilize the protonated state of His-392 during phosphoenzyme formation (Lin et al., 1992a). Mutation of Glu-327 to Ala, Gln, or Asp also reduced activity by as much as 50-fold and decreased phosphoenzyme formation by at least 1,000-fold (Lin et al., 1992a). His-392 functions as the proton donor to the leaving group, Fru 6-P (Tauler et al., 1990), and its mutation to Ala decreased activity 50–100-fold and decreased the rate of phosphoenzyme formation 1,500-fold (Lin et al., 1992a).

The different substrate specificities of members of this phosphatase/mutase family have been hypothesized to result from differences in the protein surface loops (Fig. 4). One of the yeast PGM loops, residues 92–145, forms a loosely packed lobe almost devoid of conventional secondary structure that overhangs the active-site crevice and is important in substrate binding (Winn et al., 1981). This region mapped to a 25-amino acid

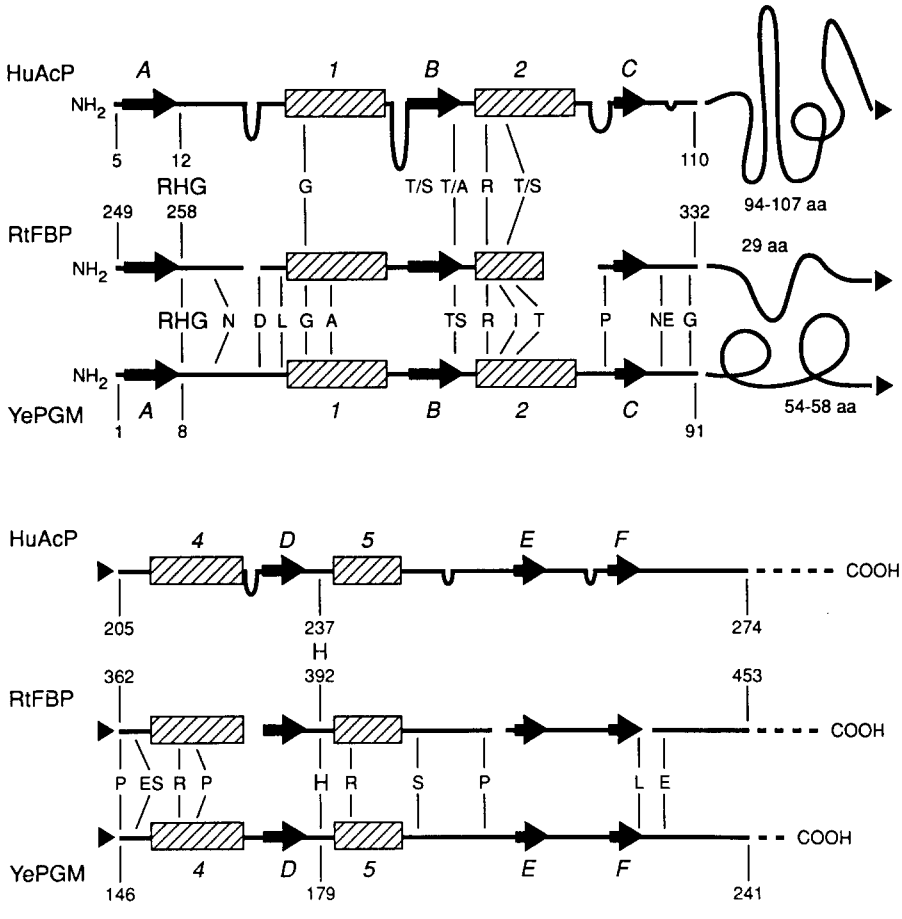

Fig. 4. Alignment of rat liver 6PF-2-K/Fru-2,6-P_2ase (RtFBP), yeast phosphoglycerate mutase (YePGM), and human prostatic acid phosphatase (HuAcP). Solid arrows, β-strands labeled A–F; hatched boxes, helices labeled numerically. Conserved residues in all three structures are shown. Yeast phosphoglycerate mutase numbering system is followed. Different lengths of surface loops in each structure refer to varying lengths found in the particular family of enzymes (mutase or acid phosphatase).

shorter chain in liver 6PF-2-K, residues 333–361, that contains three basic residues (Arg-352, Lys-356, and Arg-360) important in substrate binding. Mutation of Lys-356 to alanine increased both the K_m for Fru-2,6-P_2 and the K_i for Fru-6-P 3,000-fold without affecting the K_i for P_i (Li et al., 1992a). In addition, this mutant did not exhibit substrate inhibition. Mutation of Arg-352 to alanine produced the same changes as the Lys-356 mutant (Li et al., 1992c). These results indicate that this surface loop participates in forming the Fru-2,6-P_2/Fru-6-P binding site, and that the Fru-6-P binding site is responsible for substrate inhibition and P_i activation (Li et al., 1992c).

The third basic residue of this loop, Arg-360, contacts the C-2 phosphoryl group of Fru-2,6-P_2 in the E-P·Fru-2,6-P_2 complex because mutation of this residue to alanine greatly diminished substrate inhibition (Li et al., 1992c). This residue also contributes to binding the C-2 phosphoryl group of Fru-2,6-P_2 to the free enzyme because the Arg-360 Ala mutant increased the K_m for Fru-2,6-P_2 by 10-fold and the K_i for P_i by 12-fold without affecting the V_{max} or the K_i for Fru-6-P (Li et al., 1992c).

The yeast PGM model identified two additional basic residues as important sites for substrate binding in rat liver Fru-2,6-P_2ase (Fig. 4). Arg-7 and Arg-59 in yeast PGM have been postulated to bind phosphoglycerate (Fothergill-Gilmore & Watson, 1989). The corresponding residues in liver Fru-2,6-P_2ase, Arg-257 and Arg-307, are conserved in all known Fru-2,6-P_2ases as well as PGM (Bazan et al., 1989), and mutation of these arginines in Fru-2,6-P_2ase to alanine increased both the K_m for Fru-2,6-P_2 and the K_i for P_i, but had no effect on the K_i for Fru-6-P (Lin et al., 1992b). Therefore, these residues must contact the 2-phosphoryl group of Fru-2,6-P_2. However, the two mutations had opposite effect on V_{max}; the V_{max} of Arg-257 Ala was 11-fold higher than that of the native enzyme, whereas that for Arg-307 Ala was 700-fold lower. It has been hypothesized that Arg-257 binds substrate in the ground state more strongly than in the transition state, whereas the opposite is true for Arg-307. This differential interaction can be attributed to differences in geometry and electron distribution of the substrate between the two states (Knowles, 1980; Hockney, 1990). The formation of the E-P transition state may result in reduced stabilizing interaction between Arg-257 and the phospho group, but the gain in stabilizing interaction between Arg-307 and the phospho group is apparently enough to compensate for the loss.

Even though Fru-2,6-P_2ase has a very low turnover number of 6/min, its affinity for Fru-2,6-P_2 (K_m of 5 nM) makes it a highly efficient enzyme. The catalytic efficiency, defined as k_{cat}/K_m, is 0.2×10^8 M^{-1} s^{-1}, which is close to the diffusion control limit (Fersht, 1985). Why has mammalian Fru-2,6-P_2ase been chosen to optimize binding, rather than catalysis, during evolution? A teleological rationale can be made that by optimizing binding preferentially, Fru-2,6-P_2 is maintained at a low level, which minimizes futile cycling between the substrates and products of the Fru-6-P/Fru-1,6-P_2 and the PEP/pyruvate cycles. Because Fru-2,6-P_2 functions specifically as a regulator of glycolysis and gluconeogenesis by modulating the flux

through these cycles (Fig. 1), this prevents a high degree of substrate cycling, and energy is conserved by this strategy. In addition, energy is conserved and the capacity for precise flux control is retained because the level of Fru-2,6-P_2 is efficiently regulated by phosphorylation/dephosphorylation of the bifunctional enzyme.

Regulation of 6PF-2-K/Fru-2,6-P_2ase by phosphorylation

The liver enzyme is the only one of the five tissue-specific mammalian isoforms whose activities are both affected by phosphorylation. It is phosphorylated on Ser-32, and the phosphorylation site (Arg-Arg-Arg-Gly-Ser) is a classic cAPK consensus sequence, except that there are three basic residues NH_2-terminal to the phosphorylated serine (Murray et al., 1984). The effect of phosphorylation on 6PF-2-K at physiologic pH values (7.0–7.4) is to increase the K_m for Fru-6-P 10–20 fold, without affecting the K_m for ATP. There is also a decrease (50–65%) in the V_{max} for 6PF-2-K (Kurland et al., 1992). Phosphorylation increases the V_{max} of Fru-2,6-P_2ase by two- to fourfold (Murray et al., 1984; Stewart et al., 1986) by enhancing the breakdown of the E-P intermediate, presumably by increasing the rate of dissociation of Fru-6-P from the E-P·Fru-6-P complex (Stewart et al., 1986). Phosphorylation has no effect on the K_m for Fru-2,6-P_2.

The identity of amino acid sequences of the liver and skeletal muscle isoforms outside the NH_2-terminal region suggests that the NH_2 region plays an important role in determining the activities of the bifunctional enzyme. The advantage of having two opposing reactions on a single peptide allows for coordinate regulation of the synthesis/degradation of Fru-2,6-P_2 both at the gene and covalent modification level. We have postulated that interactions between NH_2- and COOH-terminal regions of the bifunctional enzyme isoforms are responsible for the balance between the kinase and bisphosphatase activities and for determining the different kinetic properties and regulatory roles of the different isoforms. The mechanism whereby phosphorylation reciprocally regulates kinase and bisphosphatase activities is best understood for the liver isoform, which has been the most extensively studied model system for investigating bifunctional enzyme regulation by cAPK phosphorylation. The overall pattern of regulation of the hepatic isoform by cAPK phosphorylation is shown in Figure 5. As phosphate is incorporated into the liver isoform's Ser-32 cAPK phosphorylation site to its maximum of 1 mol of phosphate/mol of enzyme subunit, the 6PF-2-K activity decreases and the Fru-2,6-P_2ase activity increases. The reciprocal regulation of the liver isoform's bifunctional activities is very sensitive to the phosphorylation state of the enzyme. For example, as shown in Figure 5, the ratio of kinase to bisphosphatase activity drops rapidly even with low amounts of phosphate incorporation into the enzyme.

In order to determine whether the primary mechanism for this reciprocal regulation was the electrostatic effect of the phospho-Ser-32 and/or a conformational/allosteric effect induced by the phospho-Ser-32, mutations of the liver isoform were constructed in which the Ser-32 was replaced by aspartate or alanine (Kurland et al., 1992). Each of these mutants was then overexpressed in *Escherichia coli*, purified to homogeneity, and their kinetic properties characterized. As shown in Table 2, the substitution

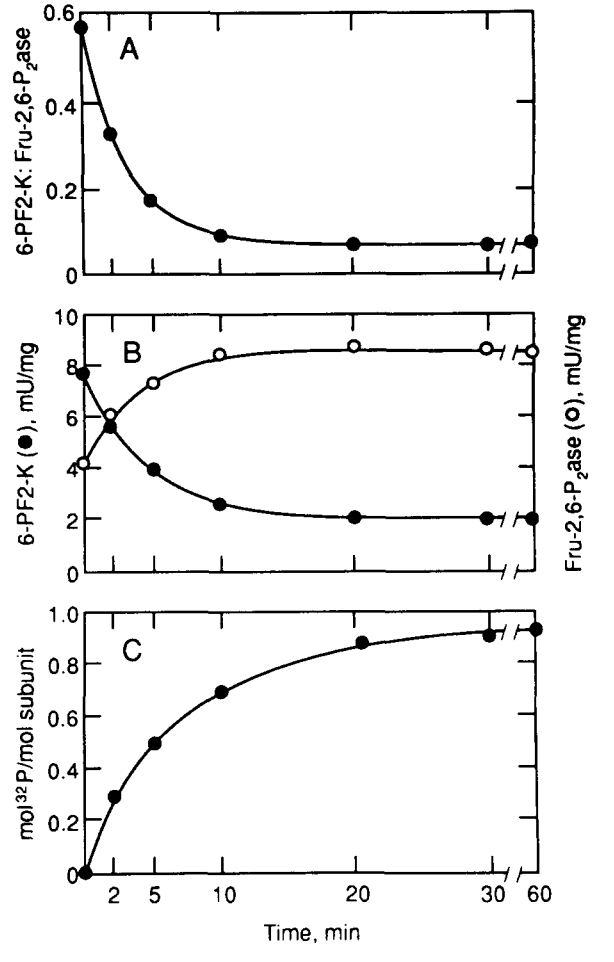

Fig. 5. Correlation between cyclic AMP-dependent phosphorylation and activities of 6PF-2-K/Fru-2,6-P_2ase. **A:** Effect of phosphorylation on the ratio of the bifunctional enzyme's two activities (6PF-2-K/Fru-2,6-P_2ase). **B:** Effect of phosphorylation on the kinase (●) and bisphosphatase (○) activities. **C:** Time course of ^{32}P incorporation into the cAPK-dependent phosphorylation site (at Ser-32) of hepatic 6PF-2-K/Fru-2,6-P_2ase.

Table 2. *Kinetic properties of 6PF-2-K/Fru-2,6-P_2ase rat liver cAPK phosphorylation-site mutants*[a]

	6PF-2-K		
Enzyme form	V^*_{max}	$S_{0.5}$ (Fru-6-P) (μM)	Fru-2,6-P_2ase V^*_{max}
WT liver (dephospho)	1.0	50	1.0
WT liver (phospho)	0.6	320	1.9
Ser-32 Ala	1.0	50	1.1
Ser-32 Ala (phospho Ser-33)	1.0	200	1.1
Ser-32 Asp	0.2	257	2.6

[a] 6PF-2-K velocity was measured in the presence of 5 mM phosphate at pH 6.4. Fru-2,6-P_2ase velocity was measured in the presence of 5 mM phosphate at pH 7.8. V^*_{max} is the maximal velocity for a given enzyme form normalized to the V_{max} of the dephosphorylated WT liver isoform. WT, wild type; V_{max}, maximal velocity; $S_{0.5}$, substrate concentration at half maximal velocity.

Table 3. *Comparisons of the effects of cAPK-catalyzed phosphorylation on 6PF-2-K activity of mutations at the Fru-6-P binding site*[a]

Enzyme form	6PF-2-K V^*_{max}	6PF-2-K $S_{0.5}$ (Fru-6-P) (μM)
WT liver (dephospho)	1.0	7
WT liver (phospho)	0.6	85
Arg-195 Ala (dephospho)	0.5	13,000
Arg-195 Ala (phospho)	0.3	19,000

[a] 6PF-2-K velocity was measured in the presence of 5 mM phosphate at pH 7.4. V^*_{max} is the maximal velocity for a given enzyme form normalized to the V_{max} of the dephosphorylated WT liver isoform. WT, wild type; V_{max}, maximal velocity; $S_{0.5}$, substrate concentration at half maximal velocity.

of aspartate for serine mimics the increase in the K_m for Fru-6-P for the phospho-form of 6PF-2-K and the increase in the V_{max} of the bisphosphatase. The substitution of alanine for Ser-32 results in nearly identical 6PF-2-K and Fru-2,6-P_2ase kinetic properties as the dephospho-form of the bifunctional enzyme. Thus, the phosphorylation effects are due to the introduction of a negatively charged group at Ser-32. Phosphorylation of a Ser-32 Ala mutant at the adjacent Ser-33 had a small effect on the kinase and no effect on the bisphosphatase, which suggests that the spatial orientation of the phosphoserine group is important for transmission of the effect.

Site-directed mutagenesis was also used to elucidate the regions and residues with which the phospho-Ser-32 may be interacting. Because phosphorylation at Ser-32 decreases Fru-6-P affinity for the kinase, it could be hypothesized that interactions of Fru-6-P with the Fru-6-P binding residue Arg-195 are affected by phosphorylation. Mutation of Arg-195 to alanine decreased the affinity of the kinase for Fru-6-P 2,000-fold (Table 3). cAPK phosphorylation at Ser-32 of the liver Arg-195 mutant had no measurable effect on the affinity of that mutant for Fru-6-P (Table 3). It can therefore be hypothesized that phosphorylation at the Ser-32 site decreases Fru-6-P affinity by affecting the interaction of Fru-6-P with Arg-195 (Kurland et al., 1993b).

In order to determine whether the structure and/or orientation of the NH_2- and COOH-terminus are important for the transmission of the effects of phosphorylation to the bisphosphatase domain of the liver isoform, mutants were produced in which the NH_2- and/or COOH-termini were partially deleted. Comparison of the Fru-6-P dependence of 6PF-2-K of the recombinantly expressed liver and skeletal muscle isoforms and of a 22-amino acid NH_2-terminal deletion of the liver isoform (ND22) indicated again that the reciprocal regulation of the bifunctional enzyme's activities is not just secondary to the electrostatic effect of the phospho-Ser-32 alone (Table 4). For example, deletion of the first 22 amino acids from the liver enzyme, to create a mutant equal in length to the native skeletal muscle isoform, increased the K_m for Fru-6-P of the kinase to a value (140 μM) that was closer to that of the muscle (340 μM) than that of the liver (7 μM). This ND22 liver mutant still retained the cAPK-dependent phosphorylation site, and phosphorylation produced changes in kinase activity that were qualitatively similar to the native liver enzyme: a decrease in the V_{max} and an increase in the K_m for Fru-6-P. Quantitatively, however, the phosphorylation-induced increase in the K_m for Fru-6-P was much less for this mutant (3-fold) in comparison to wild type (12-fold) (Kurland et al., 1993b). The bisphosphatase activity of the recombinantly expressed skeletal muscle isoform and ND22 deletion mutant were increased by fivefold. Phosphorylation resulted in only a small increase in bisphosphatase activity of the ND22 form (30%) because its activity was already elevated (Kurland et al., 1993b). The results indicate that the first 22 amino acids of the NH_2-terminus are important in (1) mediating an inhibition of the bisphosphatase, and the relief of this inhibition by Ser-32 phosphorylation; (2) enhancing Fru-6-P affinity for the kinase; and (3) mediating the control of Fru-6-P affinity of the kinase by phosphorylation.

Deletional mutagenesis also defined the role of the COOH-terminus in the response to cAPK phosphorylation. Deletion of the last 10 amino acid residues of the COOH-terminus had little effect on the activities of the bifunctional enzyme (Table 5). Deletion of the last 30 amino acids from the COOH-terminus of the liver isoform, however, reduced the kinase V_{max} by 50%, increased the K_m for Fru-6-P by twofold, and increased the V_{max} of the bisphosphatase ninefold (Table 5). Phosphorylation of this mutant decreased the kinase V_{max} and further increased the K_m for Fru-6-P by threefold but had no

Table 4. *Comparisons of the liver and skeletal muscle isoforms and the effects of cAPK-catalyzed phosphorylation on the 6PF-2-K activity of the liver isoform and its ND22 truncation mutant*[a]

Enzyme form	6PF-2-K V^*_{max}	6PF-2-K $S_{0.5}$ (Fru-6-P) (μM)	Fru-2,6-P_2ase V^*_{max}	Fru-2,6-P_2ase/6PF-2-K V^*_{max} ratio
WT liver (dephospho)	1.0	7.0	1.0	1.0
WT liver (phospho)	0.6	85	1.7	2.4
Liver ND22	0.75	140	7.2	8.5
WT skeletal muscle	0.6	330	5.0	8.3

[a] 6PF-2-K velocity and Fru-2,6-P_2ase velocity were measured in the presence of 5 mM phosphate at pH 7.4. V^*_{max} is the maximal velocity for a given enzyme form normalized to the V_{max} of the dephosphorylated WT liver isoform. WT, wild type; V_{max}, maximal velocity; $S_{0.5}$, substrate concentration at half maximal velocity.

Table 5. *Comparisons of the effects of cAPK-catalyzed phosphorylation on the 6PF-2-K activity of the liver isoform and its CD10 and CD30 truncation mutants*[a]

Enzyme form	6PF-2-K V^*_{max}	6PF-2-K $S_{0.5}$ (Fru-6-P) (μM)	Fru-2,6-P_2ase V^*_{max}	Fru-2,6-P_2ase/6PF-2-K V^*_{max} ratio
WT liver (dephospho)	1.0	7.0	1.0	1.0
WT liver (phospho)	0.6	85	1.7	2.4
Liver CD10 (dephospho)	1.0	8.0	1.0	1.0
Liver CD10 (phospho)	0.76	25	2.5	3.2
Liver CD30 (dephospho)	0.5	15	9.0	18
Liver CD30 (phospho)	0.33	50	9.0	27

[a] 6PF-2-K velocity and Fru-2,6-P_2ase velocity were measured in the presence of 5 mM phosphate at pH 7.4. V^*_{max} is the maximal velocity for a given enzyme form normalized to the V_{max} of the dephosphorylated WT liver isoform. WT, wild type; V_{max}, maximal velocity; $S_{0.5}$, substrate concentration at half maximal velocity.

effect on the elevated bisphosphatase activity (Lin et al., 1994). These results indicate that the final 30 amino acid residues of the COOH-terminus function mainly to inhibit the bisphosphatase. Phosphorylation at Ser-32 partially relieves this inhibition because the bisphosphatase is only activated two- to fourfold upon cAPK phosphorylation compared to the ninefold activation exhibited by the CD30 COOH-terminal truncated form.

Combining ND22 and CD30 COOH-terminal deletions of the rat liver enzyme in one mutant created a 418-amino acid catalytic core that still retained its cAPK-dependent phosphorylation site (Kurland et al., 1995a). The bisphosphatase of the ND22CD30 mutant was also activated ninefold, similar to the activation seen with the CD30 truncation mutant alone (Table 6). In comparison to the ND22 mutant, ND22CD30 had a similar decrease in the kinase V_{max} and a similar increase in the K_m for Fru-6-P (Kurland et al., 1995a). However, cAPK-catalyzed phosphorylation of the ND22CD30 mutant resulted in no further effect on either the kinase or the bisphosphatase, in contrast to either the ND22 or the CD30 mutant (Kurland et al., 1995a). This strongly supports the hypothesis that the effects of phosphorylation are mediated by interactions of the NH_2- and COOH-termini of the protein. The ratio of kinase/bisphosphatase maximal velocities of this catalytic core are less than the wild-type liver isoform by at least an order of magnitude, which demonstrates that the catalytic core is primarily a bisphosphatase (Table 1).

Further support for NH_2 and COOH interactions with the kinase and bisphosphatase domains, respectively, as well as with each other, is seen from bisphosphatase inhibition studies utilizing a peptide that comprises the final 30 amino acids of the COOH-terminal tail. As shown in Table 7, activation of the bisphosphatase of the ND22, CD30, and ND22CD30 forms was completely blocked by incubation of these truncated forms with the peptide. However, the peptide had no effect on the bisphosphatase activity of the intact bifunctional enzyme. This suggests that, in the absence of an intact NH_2-terminus, the COOH-terminus may relocate to a region where it cannot interact with the bisphosphatase active site.

The NH_2- and COOH-terminal interactions in the liver isoform also affected the pH dependence of the bisphosphatase (Lin et al., 1994). In the dephosphorylated state, the activity of the bisphosphatase of the native rat liver isoform reaches a maximum at pH 5.0, but decreases to 12% of that at pH 7.0–7.5. As seen in Figure 6, this decrease depended on deprotonation

Table 6. *Comparison of the effects of cAPK-catalyzed phosphorylation on the 6PF-2-K and Fru 2,6-P_2ase activities of the native liver and skeletal muscle bifunctional enzyme isoforms and of various truncated enzyme forms*[a]

Enzyme form	6PF-2-K V^*_{max}	Fru-2,6-P_2ase V^*_{max}	Fru-2,6-P_2ase/6PF-2-K V^*_{max} ratio
WT liver (dephospho)	1.0	1.0	1.0
WT liver (phospho)	0.72	1.7	2.4
WT skeletal muscle	0.6	5.0	8.3
Liver ND22 (dephospho)	0.85	7.2	8.5
Liver ND22 (phospho)	0.33	8.0	24.2
Liver ND22CD30 (dephospho)	0.55	9.5	17.3
Liver ND22CD30 (phospho)	0.24	9.0	37.5

[a] 6PF-2-K velocity and Fru-2,6-P_2ase velocity were measured in the presence of 5 mM phosphate at pH 7.4. V^*_{max} is the maximal velocity for a given enzyme form normalized to the V_{max} of the dephosphorylated WT liver isoform. WT, wild type; V_{max}, maximal velocity; $S_{0.5}$, substrate concentration at half maximal velocity.

Table 7. *Effect of a peptide composed of the final 30 amino acids of the COOH-terminus of the bisphosphatase domain on inhibition of Fru-2,6-P_2ase activity*

	Fru-2,6-P_2ase activity (μmol/min/mg enzyme)	
Enzyme form	No peptide	+Peptide
WT liver	10	10
CD30	100	13
ND22	60	12
ND22CD30	64	11

of a group with a pK of 5.7. In contrast, the pH dependence of the bisphosphatase for the skeletal muscle isoform, the separately expressed bisphosphatase domain, and the liver CD30 truncated form exhibited pH-insensitive V_{max} values that were three- to ninefold higher at pH 7.0–7.5 than that of the bisphosphatase of the native liver form (Lin et al., 1994). cAPK-catalyzed phosphorylation of the liver isoform activated the bisphosphatase, an effect known to enhance the rate of E-P breakdown (Stewart et al., 1986). The phospho-liver isoform has a V_{max} versus pH profile that was shifted toward higher pH when compared to that of the dephospho-form (Fig. 6), indicating that a group with a pK_a of 5.7 in the pH titration of the dephospho-form was increased to 6.4 in the phospho-form. Phosphorylation of the liver CD30 mutant had no effect on the bisphosphatase V_{max} (Table 5). The separately expressed bisphosphatase domain has a 10-fold activation of its V_{max}, and a CD30 truncation of the separately expressed bisphosphatase domain did not result in any further bisphosphatase activation (Table 5). These data support the observations derived from the kinetic studies of the liver ND22 form that indicate the NH_2-terminus inhibits the bisphosphatase (Kurland et al., 1993b). The data also indicate that NH_2- and COOH-terminal interactions in the liver bifunctional enzyme affect both pH dependence of the Fru-2,6-P_2ase and its activation by phosphorylation. The pH kinetics of various enzyme forms revealed two distinct Fru-2,6-P_2ase activity states. The higher activity state is characterized by an insensitivity of V_{max} to pH, whereas the low activity state is characterized by about an order of magnitude decrease in V_{max} as the pH increases from 5 to 10. It may be that the group with a pK_a of 5.7 in the liver isoform is shifted to a value greater than 10 in the pH-insensitive bisphosphatase forms (CD30, separate bisphosphatase domain, skeletal muscle isoform). In the physiological pH range, this group exists mostly in the deprotonated state, and the maximal velocity of the Fru-2,6-P_2ase is about eightfold less than the optimum value. Hence, the protonated state of this group is not essential for activity but is responsible for fine tuning the Fru-2,6-P_2ase activity in a pH-dependent manner.

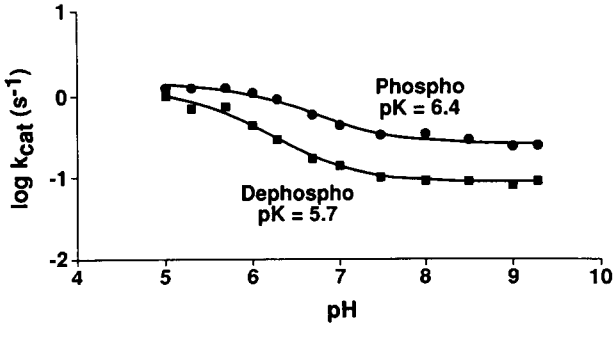

Fig. 6. Effect of cAMP-dependent protein kinase-catalyzed phosphorylation of hepatic 6PF-2-K/Fru-2,6-P_2ase on the pH dependence of k_{cat}. Lines through the points represent the theoretical curves. ■, Dephosphorylated enzyme; ●, phosphorylated enzyme.

The kinetic properties of other 6PF-2-K/Fru-2,6-P_2ase isoforms also seems dependent on the structure of the NH_2- and COOH-terminal ends. For example, deletion of 24 and 30 amino acids from the NH_2-terminus of the testis isoform resulted in a decreased affinity for Fru-6-P, a decreased kinase V_{max}, and a twofold increase in bisphosphatase activity (Tominaga et al., 1993). The brain isoform, which has extensive NH_2- and COOH-termini outside of catalytic core such that the brain enzyme is twice the size (110 kDa) of the liver form (55 kDa), is phosphorylated by cAMP-dependent protein kinase. However, this phosphorylation has no effect on the kinase and bisphosphatase activities (Ventura et al., 1992), perhaps because the terminal interactions are unaffected.

Yeast PFK26 (Fig. 2) is similar in size to the brain isoform and does undergo a cAMP-dependent phosphorylation-induced 6PF-2-K activity change. Phosphorylation at the consensus phosphorylation site (RRYS, Ser-644) at the COOH-terminal end of the Fru-2,6-P_2ase domain causes kinase activation (Francois et al., 1988). This is analogous to the heart enzyme.

The extended COOH-terminal region of the heart isoform appears to interact with the bisphosphatase domain to inhibit activity and/or prevent E-P formation. When the entire human heart Fru-2,6-P_2ase domain was expressed in bacteria, it was devoid of activity and did not form a phosphoenzyme intermediate (Lange & Pilkis, 1995). However, truncation of this form to a size that corresponds to the liver Fru-2,6-P_2ase domain resulted in an active form that did form a phosphoenzyme intermediate (Lange & Pilkis, 1995).

The response of the 58-kDa heart isoform to cAPK phosphorylation also differs dramatically from that of the liver isoform. cAPK-catalyzed phosphorylation at Ser-466 activates the kinase by decreasing the K_m for Fru-6-P by 50% (Kitamura et al., 1988; Rider et al., 1992b), rather than the large increase in the K_m for Fru-6-P seen for the liver form upon cAPK phosphorylation at Ser-32 (see below). No effects of phosphorylation on Fru-2,6-P_2ase activity of the heart form have been detected, perhaps because its activity is very low at physiological pH (El-Maghrabi et al., 1986; Rider et al., 1992b). Rat heart 6PF-2-K/Fru-2,6-P_2ase is a substrate for both cAMP-dependent protein kinase and the Ca^{2+}/camodulin-dependent protein kinase (Depre et al., 1993). Both protein kinases catalyze phosphorylation of Ser-466 and Ser-483, which decreases the K_m for Fru-6-P by 50%.

The cAPK-catalyzed increase in heart kinase activity is consistent with the metabolic exigencies of heart tissue. Epinephrine-induced increases in cAMP levels and heart contractility would be expected to increase glycolytic flux. An increase in the workload of the heart has been shown to correlate with a decrease in the K_m for Fru-6-P for 6PF-2-K measured in rat heart extracts (Depre et al., 1993). They attributed this to phosphor-

ylation by Ca^{2+}/calmodulin-dependent protein kinase, but other possibilities could not be excluded. The effect of cAPK-dependent phosphorylation on the heart isoform is rather small, amounting to at most a 50% reduction in the K_m for Fru-6-P. The correlation of heart work load with the decrease in the K_m for Fru-6-P (Depre et al., 1993) does not necessarily imply causation. Additional studies on the effects of phosphorylation of the heart isoform await development of systems expressing it in high yield.

Regulation of 6PF-2-K/Fru-2,6-P_2ase by ADP-ribosylation

Hepatic 6PF-2-K/Fru-2,6-P_2ase is ADP-ribosylated by arginine-specific ADP-ribosyl transferase (Rosa et al., 1995). All three arginine residues in the cAMP-dependent phosphorylation site sequence can be ADP-ribosylated, but it occurs preferentially at Arg-29 and Arg-30. ADP-ribosylation of 6PF-2-K/Fru-2,6-P_2ase blocked its phosphorylation by cAPK and decreased its kinase activity and recognition by anti-6PF-2-K/Fru-2,6-P_2ase antibodies but did not alter Fru-2,6-P_2ase activity. ADP-ribosylation only affects the kinase domain and perhaps does not affect NH_2- and COOH-terminal interactions, in contrast to cAPK-catalyzed phosphorylation in the same area. ADP-ribosylation may constitute an additional posttranslational regulatory mechanism of hepatic 6PF-2-K/Fru-2,6-P_2ase, but its physiologic relevance remains uncertain. One situation where this may occur is during liver regeneration, where the enzyme did not respond to glycerol-3-phosphate, was not affected by cAMP-dependent phosphorylation, and was not recognized by a liver-specific antibody (Rosa et al., 1990).

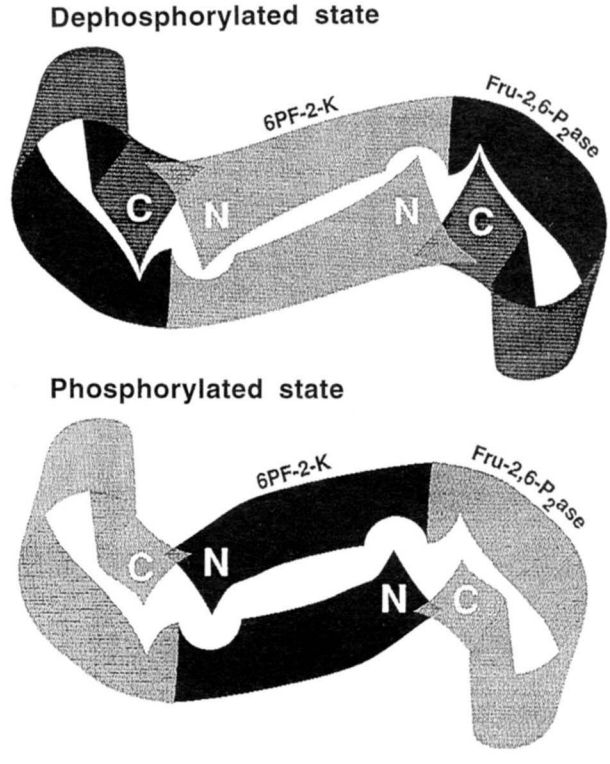

Fig. 7. Model illustrating the effect of Ser-32 phosphorylation (by cAPK) on NH_2- and COOH-termini interactions of the hepatic bifunctional enzyme. The bifunctional enzyme is postulated to exist as a homodimer with an antiparallel configuration. NH_2- and COOH-termini interact separately with the active sites of their respective domains, as well as with the opposing terminus in the other domain. In the dephosphorylated state, the kinase domain is activated (gray) and the bisphosphatase domain is inhibited (black). Phosphorylation at Ser-32 causes a restructuring of the interaction between these NH_2- and COOH-termini and their respective active sites. This results in an inhibition of the kinase domain (black) and an activation of the bisphosphatase domain (gray).

Summary

Experimental evidence supports the hypothesis that the NH_2- and COOH-terminal regions of the liver bifunctional enzyme interact individually with the kinase and bisphosphatase domains, respectively (Kurland et al., 1993b; Lin et al., 1994). The termini also appear to interact with their counterparts in the other subunit. A model has been developed to explain how the NH_2- and COOH-termini interactions mediate the effect of phosphorylation on the liver bifunctional enzyme. As shown in Figure 7, the bifunctional homodimer is postulated to exist in an antiparallel configuration, with the NH_2- and COOH-termini regions interacting separately with the active sites of their respective domains, as well as interacting with the opposing terminus in the other domain in both the phosphorylated and dephosphorylated states. Deletion of either the NH_2- or COOH-terminal regions, or both regions simultaneously, leads to disruption of the NH_2- and COOH-terminal interactions, resulting in a 5–10-fold activation of the bisphosphatase. Phosphorylation of Ser-32 may cause a restructuring, or uncoupling, of the interaction between these NH_2- and COOH-termini, but it is evident that there is still an interaction because the bisphosphatase is activated to a greater degree than by phosphorylation by either a deletion of the first 22 NH_2-terminal or last 30 COOH-terminal residues.

The mechanism of the interaction of the NH_2- and COOH-termini with each other, and with the active sites of the bifunctional enzyme, can only be speculated upon because crystallization of the native bifunctional enzyme has not been achieved. It is reasonable to propose that the NH_2-terminal region is exposed on the surface of the 6PF-2-K subunit because partial thermolysin digestion rapidly removes the Ser-32 phosphorylation site and activates the bisphosphatase (El-Maghrabi et al., 1984a). This result is consistent with the postulated role of the NH_2-terminal region moving to a new location upon phosphorylation. The observation that the shift in the Fru-6-P affinity of 6PF2-K with phosphorylation or deletion of the first 22 NH_2-terminal residues, in addition to the observation that the liver isoform has a higher V_{max} than any of the truncated forms, suggests that this region may form part of the kinase active site. Because the major effect of phosphorylation on the 6PF-2-K domain is to decrease the affinity for Fru-6-P, with a secondary effect of mildly decreasing the kinase V_{max}, another possible regulatory mechanism is that the NH_2-terminus of the liver isoform regulates access of the substrate to the kinase active site. This possibility would be analogous to the proposed mechanism of regulation of yeast glycogen phosphorylase, whose structure in the nonphosphorylated form has recently been solved by Rath and Fletterick (1994).

It has been proposed that phosphorylation activates that enzyme by causing the phospho-NH_2-terminus to be repelled from an orientation, which impedes the opening to the catalytic site. Phosphorylation causes refolding such that the enzyme

adopts the fully active conformation, a mechanism termed relief of steric and conformational blocking (Johnson, 1994). Proteolytic cleavage of the NH_2-terminal residues of yeast phosphorylase also results in activation (Becker et al., 1983), indicating that relief of this blocking mechanism can be achieved by total removal of these NH_2-terminal residues, or by their relocation secondary to phosphorylation. An analogy may be drawn to the contribution of the NH_2-terminus to the Fru-6-P affinity of the 6PF-2-K domain, and the regulation of Fru-6-P affinity by phosphorylation. The high-affinity state of 6PF-2-K occurs in the dephospho-state of the liver isoform and is dependent on the presence of the initial portion of the NH_2-terminus. Phosphorylation of the liver isoform at Ser-32 or deletion of the first 22 amino acids results in a low Fru-6-P affinity state. The NH_2-terminus of the bifunctional enzyme may facilitate access of Fru-6-P to the 6PF-2-K catalytic site, and this access may be impeded upon cAPK-catalyzed phosphorylation at Ser-32.

Although removal of the initial 22 NH_2-terminal amino acids of the bifunctional enzyme activates the bisphosphatase more than cAPK phosphorylation, the NH_2-terminus may not inhibit the bisphosphatase directly. It seems more likely that the COOH-terminus inhibits the bisphosphatase activity directly, due to autoinhibition by the COOH-terminal tail, or indirectly through a steric and conformational blocking mechanism. Autoinhibition seems more likely because a peptide composed of the COOH-terminal 30 amino acids inhibits the bisphosphatase activity of liver isoforms truncated at the NH_2-terminus (ND22) and/or the COOH-terminus (ND22CD30 and CD30). The NH_2-terminus may help promote inhibitory interactions between the COOH-terminus and the bisphosphatase catalytic site. This hypothesis is supported by the observations that (1) the bisphosphatase activity is the same for both the separately expressed bisphosphatase domain (residues 251–470) and a 30-amino acid COOH-terminal deletion of the bisphosphatase domain (residues 251–440); (2) the COOH-terminal 30-amino acid peptide inhibits the ND22 mutant, which has an intact COOH-terminus. Thus, if the NH_2-terminus is not intact and present, the COOH-terminal tail may move out of the bisphosphatase active site.

Autoinhibition of the bisphosphatase by the COOH-terminus has parallels in the self-regulation of several protein kinases, such as the family typified by cAMP-dependent protein kinase and twitchin kinase (Kemp et al., 1994). Recently, X-ray crystallographic analysis of a catalytic fragment of twitchin kinase (amino acids 5890–6262) revealed that autoinhibition was mediated by the interaction of the final 60 amino acids in the COOH-terminal tail with a region near the catalytic site. Truncation of the final 19 amino acids (to Leu-6244) in this twitchin kinase fragment resulted in activation (Lei et al., 1994), which is analogous to the effect of COOH-terminal truncation of Fru-2,6-P_2ase. The catalytic site region of twitchin kinase had a hinge region characterized, in part, by adjacent glycine residues in proximity to an ATP-binding sequence. Inspection of the bifunctional enzyme reveals such a motif near the bisphosphatase active site (Gly_{267} Arg Ile **Gly Gly** Asp Ser Gly_{274}). It may be that phosphorylation of the liver isoform attenuates autoinhibition of the bisphosphatase, as well as inhibiting the kinase. This hypothesis implies that the two active sites of the bifunctional enzyme lie near each other because the termini are postulated to always have some interaction in both the phosphorylated and dephosphorylated states and still be able to interact with the active sites of their respective domains. The NH_2- and COOH-termini of the various bifunctional enzyme forms may modulate the ratio of the activities of the catalytic core, which is primarily a bisphosphatase, through similar mechanisms.

In conclusion, it has been proposed that a combination of electrostatic, allosteric, and autoregulatory effects are involved in the reciprocal regulation of the bifunctional enzyme's activities. Allosteric control mechanisms must be involved after cAPK-catalyzed phosphorylation of the bifunctional enzyme, by definition (reviewed in Kemp & Pearson, 1991). This allosteric control is manifest in the relief of the autoinhibitory influence of the COOH-terminal tail for the bisphosphatase after phosphorylation at the NH_2-terminus. Also, the subsequent decrease in Fru-6-P affinity for the kinase domain after phosphorylation at Ser-32, which is attenuated in the liver ND22 mutant, implies both allosteric and electrostatic kinase regulatory mechanisms. The crystallographic structure of the activated form of the bisphosphatase domain with a truncation of 30 amino acids at the COOH-terminus has been solved (Lee et al., 1995). In the future, crystallization of this activated form with the 30-amino acid COOH-terminal peptide will be indispensable in proving that COOH-terminal inhibition occurs at the active site, as required by the intrasteric inhibition model (Kemp & Pearson, 1991). Crystallization and comparison of the structures of all bifunctional enzyme isoforms should result in new insights into the general mechanism of enzyme regulation by phosphorylation, as well as for autoregulatory mechanisms of kinases.

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References

Abe Y, Uyeda K. 1994. Effect of adding phosphorylation sites for cAMP-dependent protein kinase to rat testis 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Biochemistry 33*:5766–5771.

Bazan JF, Fletterick RJ, Pilkis SJ. 1989. The evolution of a enzyme: 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Proc Natl Acad Sci USA 86*:9642–9646.

Becker JO, Wingender-Drissen R, Schiltz E. 1983. Purification and properties of phosphorylase from baker's yeast. *Arch Biochem Biophys 225*:667–678.

Benkovic S. 1992. Catalytic antibodies. *Annu Rev Biochem 61*:29–54.

Berger SA, Evans PR. 1990. Active-site mutants altering the cooperativity of *E. coli* phosphofructokinase. *Nature 343*:575–576.

Branden C, Tooze J. 1991. *Introduction to protein structure*. New York/London: Garland Publishing Inc.

Branden CI. 1980. Relation between the structure and function of alpha/beta-proteins. *Q Rev Biophys 13*:317–332.

Claus TH, Schlumpf JR, El-Maghrabi MR, Pilkis SJ. 1982. Regulation of the phosphorylation and activity of 6-phosphofructo-1-kinase in isolated hepatocytes by α-glycerolphosphate and fructose-2,6-bisphosphate. *J Biol Chem 257*:7542–7550.

Colosia AD, Lively M, El-Maghrabi MR, Pilkis SJ. 1987. Isolation of a cDNA clone for rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Biochem Biophys Res Commun 143*:1092–1098.

Crepin KM, Darville MI, Hue L, Rousseau GG. 1989. Characterization of distinct mRNAs coding for putative isoenzymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Eur J Biochem 183*:433–440.

Crepin KM, De Cloedt M, Vertommen D, Foret D, Michel A, Rider MH, Rousseau GG, Hue L. 1992. Molecular forms of 6-phosphofructo-2-

kinase/fructose-2,6-bisphosphatase expressed in rat skeletal muscle. *J Biol Chem 267*:21698–21704.

Darville MI, Crepin KM, Hue L, Rousseau GG. 1989. 5′ Flanking sequence and structure of a gene encoding rat 6-phospho-fructo-2-kinase/fructose-2,6-bisphosphatase. *Proc Natl Acad Sci USA 86*:6543–6547.

Darville MI, Crepin KM, Vandekerckhove J, Vandamme J, Octave JN, Rider MH, Hue L, Rousseau GG. 1987. Complete nucleotide sequence coding for rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase derived from a cDNA clone. *FEBS Lett 224*:317–321.

Dean AM, Koshland DE Jr. 1990. Electrostatic and steric contributions to regulation at the active site of isocitrate dehydrogenase. *Science 249*: 1044–1046.

Depre C, Rider M, Veitch K, Hue L. 1993. Role of fructose-2,6-bisphosphate in the control of heart glycolysis. *J Biol Chem 268*:13274–13279.

El-Maghrabi MR, Claus TH, Pilkis J, Pilkis SJ. 1981. Partial purification of a rat liver enzyme that catalyzes the formation of fructose-2,6-bisphosphate. *Biochem Biophys Res Commun 101*:1071–1077.

El-Maghrabi MR, Correia JJ, Heil P, Pate T, Cobb C, Pilkis SJ. 1986. Tissue distribution, immunoreactivity, and physical properties of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Proc Natl Acad Sci USA 83*:5005–5009.

El-Maghrabi MR, Fox E, Pilkis J, Pilkis SJ. 1982a. Cyclic AMP-dependent phosphorylation of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Biochem Biophys Res Commun 106*:794–802.

El-Maghrabi MR, Pate TM, Murray KJ, Pilkis SJ. 1984a. Differential effects of proteolysis and protein modification on the activities of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J Biol Chem 259*:13096–13103.

El-Maghrabi MR, Pate TM, Pilkis SJ. 1984b. Characterization of the exchange reactions of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Biochem Biophys Res Commun 123*:749–756.

El-Maghrabi MR, Pilkis J, Fox E, Claus TH, Pilkis SJ. 1982b. Regulation of rat liver fructose-2,6-bisphosphate. *J Biol Chem 257*:7603–7607.

Fersht A. 1985. *Enzyme structure and function*. New York: W.H. Freeman and Company.

Fothergill-Gilmore LA, Watson HC. 1989. The phosphoglycerate mutases. *Adv Enzymol 62*:227–313.

Francois J, Van Schaftingen E, Hers HG. 1988. Characterization of phosphofructokinase 2 and of enzymes involved in the degradation of fructose-2,6-bisphosphate in yeast. *Eur J Biochem 171*:599–608.

Heierhorst J, Probst WC, Vilim FS, Buku A, Weiss KR. 1994. Autophosphorylation of molluscan twitchin and interaction of its kinase domain with calcium/calmodulin. *J Biol Chem 269*:21086–21093.

Hellinga HW, Evans PR. 1985. Nucleotide sequence and high level expression of the major *Escherichia coli* phosphofructokinase. *Eur J Biochem 149*:363–373.

Hockney DD. 1990. Enzyme binding and catalysis. In: Sigmon DS, Boyer PD, eds. *The enzymes, vol 19*. New York: Academic Press, Inc. pp 1–36.

Hu SH, Parker MW, Lei JY, Wilce MCJ, Benian GM, Kemp BE. 1994. Insights into autoregulation from the crystal structure of twitchin kinase. *Nature 369*:581–584.

Hurley JH, Dean AM, Sohl JL, Koshland DE Jr, Stroud RM. 1990. Regulation of an enzyme by phosphorylation at the active site. *Science 249*: 1012–1016.

Johnson LH. 1992. Glycogen phosphorylase: Control by phosphorylation and allosteric factors. *FASEB J 6*:2274–2282.

Johnson LH. 1994. Control by protein phosphorylation. *Nature Struct Biol 1*:657–659.

Kemp BE, Parker MW, Hu S, Tiganis T, House C. 1994. Substrate and pseudosubstrate interactions with protein kinases: Determinants of specificity. *Trends Biochem Sci 19*:440–444.

Kemp BE, Pearson RB. 1991. Intrasteric regulation of protein kinases and phosphatases. *Biochim Biophys Acta 1094*:67–76.

Kitajima S, Sakakibara R, Uyeda K. 1984. Kinetic studies of fructose 6-phosphate,2-kinase and fructose-2,6-bisphosphatase. *J Biol Chem 259*:6896–6903.

Kitamura K, Kangawa K, Matuso H, Uyeda K. 1988. Phosphorylation of myocardial fructose-6-phosphate,2-kinase: Fructose-2,6-bisphosphatase by cAMP-dependent protein kinase and protein kinase C. Activation by phosphorylation and amino acid sequences of the phosphorylation sites. *J Biol Chem 263*:16796–16801.

Kitamura K, Uyeda K. 1987. The mechanism of activation of heart fructose 6-phosphate,2-kinase: Fructose-2,6-bisphosphatase. *J Biol Chem 262*: 679–681.

Kitamura K, Uyeda K, Kangawa K, Matsuo M. 1989. Purification and characterization of rat skeletal muscle fructose-6-phosphate,2-kinase: Fructose-2,6-bisphosphatase. *J Biol Chem 264*:9799–9806.

Knighton DR, Pearson RB, Sowadski JM, Means AR, Ten Eyck LF, Taylor SS, Kemp BE. 1992. Structural basis of the intrasteric regulation of myosin light chain kinases. *Science 258*:130–135.

Knowles JR. 1980. Enzyme-catalyzed phosphoryl transfer reactions. *Annu Rev Biochem 49*:877–919.

Kountz PD, Freeman S, Cook AG, El-Maghrabi MR, Knowles JR, Pilkis SJ. 1988. The stereochemical course of phospho group transfer catalyzed by rat liver 6-phosphofructo-2-kinase. *J Biol Chem 263*:16069–16072.

Kountz PD, McCain RW, El-Maghrabi MR, Pilkis SJ. 1986. Hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: Phosphate dependence and effect of other oxyanions. *Arch Biochem Biophys 251*: 104–113.

Kretschmer K, Fraenkel DG. 1991. Yeast 6-phosphofructo-2-kinase: Sequence and mutant. *Biochemistry 30*:10663–10672.

Kurland IJ, El-Maghrabi MR, Correia J, Pilkis SJ. 1992. Rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: Properties of the phospho- and dephospho-forms and of two mutants in which Ser^{32} has been changed by site-directed mutagenesis. *J Biol Chem 267*:4416–4423.

Kurland IJ, Chapman B, El-Maghrabi MR, Pilkis SJ. 1995. Characterization of the catalytic core of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: Evidence for an intrasteric autoregulatory mechanism. *Proc Natl Acad Sci USA*. Forthcoming.

Kurland IJ, El-Maghrabi MR, Pilkis SJ. 1993a. Lack of evidence for a role of Cys-138 as a base catalyst in the skeletal muscle 6-phospho-fructo-2-kinase reaction. *Biochem Biophys Res Commun 195*:228–236.

Kurland IJ, Li L, Lange AJ, Correia JJ, El-Maghrabi MR, Pilkis SJ. 1993b. Regulation of rat 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J Biol Chem 268*:14056–14064.

Lange AJ, El-Maghrabi MR, Pilkis SJ. 1991. Isolation of bovine liver 6-phospho-fructo-2-kinase/fructose-2,6-bisphosphatase cDNA: Bovine liver and heart forms of the enzyme are separate gene products. *Arch Biochem Biophys 290*:258–263.

Lange AJ, Li L, Vargas AM, Pilkis SJ. 1993. Expression of human liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in *Escherichia coli*. Role of the N-2 proline in the degradation of the enzyme. *J Biol Chem 268*:9466–9472.

Lange AJ, Pilkis SJ. 1990. Sequence of human liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Nucleic Acids Res 18*:3652.

Lange AJ, Pilkis SJ. 1995. Characterization of the separately expressed bisphosphatase domain of heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Arch Biochem Biophys*. Forthcoming.

Lee YH, Pflugrath J, Ogata C, Pilkis SJ. 1995. Crystal structure of the separately expressed bisphosphatase domain of liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Nature Struct Biol*. Forthcoming.

Lei J, Tang X, Chambers TC, Pohl J, Benian GM. 1994. Protein kinase domain of twitchin has protein kinase activity and an autoinhibitory region. *J Biol Chem 269*:21078–21085.

Li L, Lange AJ, Pilkis SJ. 1993. Isolation of a cDNA for chicken liver 6-phospho-fructo-2-kinase/fructose-2,6-bisphosphatase. *Biochem Biophys Res Commun 190*:397–405.

Li L, Lin K, Correia JJ, Pilkis SJ. 1992a. Lysine 356 is a critical residue for binding the C-6 phospho group of fructose 2,6-bisphosphate to the fructose-2,6-bisphosphatase domain of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J Biol Chem 267*:16669–16675.

Li L, Lin K, Kurland IJ, Correia JJ, Pilkis SJ. 1992b. Site-directed mutagenesis in rat liver 6-phospho-fructo-2-kinase: Mutation at the fructose-6-phosphate binding site affects phosphate activation. *J Biol Chem 267*: 4386–4393.

Li L, Lin K, Pilkis J, Correia JJ, Pilkis SJ. 1992c. Hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. The role of surface loop basic residues in substrate binding to the fructose-2,6-bisphosphatase domain. *J Biol Chem 267*:21588–21594.

Lin K, Kurland IJ, Li L, Lee YH, Okar D, Marecek JF, Pilkis SJ. 1994. Evidence for NH_2- and COOH-terminal interactions in rat 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J Biol Chem 269*:16953–16960.

Lin K, Li L, Correia JJ, Pilkis SJ. 1992a. Glu^{327} is part of a catalytic triad in rat liver fructose-2,6-bisphosphatase. *J Biol Chem 267*:6556–6562.

Lin K, Li L, Correia JJ, Pilkis SJ. 1992b. Arg-257 and Arg-307 of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase bind the C-2 phospho group of fructose-2,6-bisphosphate in the fructose-2,6-bisphosphatase domain. *J Biol Chem 267*:19163–19171.

Lively MO, El-Maghrabi MR, Pilkis J, D'Angelo G, Colosia AD, Ciavola JA, Fraser BA, Pilkis SJ. 1988. Complete amino acid sequence of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J Biol Chem 263*:839–849.

Murray KJ, El-Maghrabi MR, Kountz PD, Lukas TJ, Soderling TR, Pilkis SJ. 1984. Amino acid sequence of the phosphorylation site of rat liver 6PF-2-K/Fru-2,6-P_2ase. *J Biol Chem 259*:7673–7681.

O'Toole GA, Trzebiatowski JR, Esialante-Semerena JC. 1995. The cobC gene of *Salmonella typhimurium* codes for a novel phosphatase involved in the assembly of the nucleotide loop of cobalamin. *J Biol Chem*. Forthcoming.

Paravicini G, Kretschmer M. 1992. The yeast FBP26 gene codes for a fructose-2,6-bisphosphatase. *Biochemistry 31*:7126–7133.

Pilkis SJ, Claus TH. 1991. Hepatic gluconeogenesis/glycolysis: Regulation and structure/function relationships of substrate cycle enzymes. *Annu Rev Nutr 11*:465–515.

Pilkis SJ, Lively MO, Pilkis J, El-Maghrabi MR. 1987. Active site sequence of hepatic fructose 2,6-bisphosphatase. *J Biol Chem 262*:12672–12675.

Pilkis SJ, Pilkis J, El-Maghrabi MR. 1985. The sugar phosphate specificity of rat hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J Biol Chem 260*:7551–7556.

Rath VL, Fletterick RJ. 1994. Parallel evolution in two homologues of phosphorylase. *Nature Struct Biol 1*:681–690.

Rider MH, Vandamme J, Lebeau E, Vertommen D, Vidal H, Rousseau GG, Vandekerckhove J, Hue L. 1992a. The two forms of bovine heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase result from alternative splicing. *Biochem J 285*:405–411.

Rider MH, Vandamme J, Vertommen D, Michael A, Vandekerckhove J, Hue L. 1992b. Evidence for new phosphorylation sites for protein kinase C and cyclic AMP-dependent protein kinase in bovine heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *FEBS Lett 310*: 139–142.

Rosa JL, Perez X, Ventura F, Tauler A, Gil J, Shimoyama M. 1995. ADP-ribosylation of hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. 1995. *Biochem J*. Forthcoming.

Rosa JL, Ventura F, Carreras J, Bartrons R. 1990. Fructose 2,6-bisphosphate and 6-phosphofructo-2-kinase during liver regeneration. *Biochem J 270*: 645–649.

Sakata J, Abe Y, Uyeda K. 1991. Molecular cloning of the DNA and characterization of rat testes fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase. *J Biol Chem 266*:15764–15770.

Sakata J, Uyeda K. 1990. Bovine heart fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase: Complete amino acid sequence and localization of phosphorylation sites. *Proc Natl Acad Sci USA 87*:4951–4955.

Schirmer T, Evans PR. 1990. Structural basis of the allosteric behaviour of phosphofructokinase. *Nature 343*:140–145.

Shirakihara Y, Evans PR. 1988. Crystal structure of the complex of phosphofructokinase from *Escherichia coli* with its reaction products. *J Mol Biol 204*:973–994.

Soderling TR. 1990. Protein kinases: Regulation by autoinhibitory domains. *J Biol Chem 265*:1823–1826.

Stewart HB, El-Maghrabi MR, Pilkis SJ. 1985. Evidence for a phosphoenzyme intermediate in the reaction pathway of rat hepatic fructose-2,6-bisphosphatase. *J Biol Chem 260*:12935–12941.

Stewart HB, El-Maghrabi MR, Pilkis SJ. 1986. Mechanism of activation of fructose-2,6-bisphosphatase by cAMP-dependent protein kinase. *J Biol Chem 261*:8793–8798.

Tauler A, El-Maghrabi MR, Pilkis SJ. 1987. Functional homology of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, phosphoglycerate mutase, and 2,3-bisphosphoglycerate mutase. *J Biol Chem 262*: 16808–16815.

Tauler A, Lin K, Pilkis SJ. 1990. Hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: Use of site-directed mutagenesis to evaluate the roles of His-258 and His-392 in catalysis. *J Biol Chem 265*:15617–15622.

Taylor SS, Knighton DR, Zheng J, Sowadski JM. 1993. cAMP-dependent protein kinase: A template for the protein kinase family. *Trends Biochem Sci 18*:84–89.

Tominaga N, Minami Y, Sakakibara R, Uyeda K. 1993. Significance of the amino terminus of rat testis fructose-6-phosphate,2-kinase: Fructose-2,6-bisphosphatase. *J Biol Chem 268*:15951–15957.

Tsuchya Y, Uyeda K. 1994. Bovine heart fructose-6-phosphate,2-kinase: Fructose-2,6-bisphosphatase mRNA and gene structure. *Arch Biochem Biophys 310*:467–474.

Valdez BC, French BA, Younathan ES, Chang S. 1989. Site-directed mutagenesis in *Bacillus stearothermophilus* fructose-6-phosphate 1-kinase: Mutation at the substrate binding site affects allosteric behaviour. *J Biol Chem 264*:131–135.

Van Schaftingen E, Davies DR, Hers HG. 1981. Inactivation of phosphofructokinase 2 by cyclic AMP-dependent protein kinase. *Biochem Biophys Res Commun 103*:362–369.

Van Schaftingen E, Hers HG. 1982. Fructose-2,6-bisphosphatase from rat liver. *Eur J Biochem 124*:143–149.

Van Schaftingen E, Hers HG. 1986. Purification and properties of phosphofructokinase 2/fructose-2,6-bisphosphatase from chicken liver and from pigeon muscle. *Eur J Biochem 159*:359–365.

Ventura F, El-Maghrabi MR, Lange AJ, Pilkis SJ, Bartrons R. 1995. Molecular cloning of bovine brain 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Biochem Biophys Res Commun*. Forthcoming.

Ventura F, Rosa JL, Ambrosio S, Pilkis SJ, Bartrons R. 1992. Bovine brain 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: Evidence for a neural specific isoenzyme. *J Biol Chem 267*:17939–17943.

Winn SI, Watson HC, Harkins RN, Fothergill LA. 1981. Phosphoglycerate mutase. *Phil Trans R Soc Lond Ser B 293*:121–130.

Zheng J, Knighton DR, Ten Eyck LF, Karlsson R, Xuong NH, Taylor SS, Sowadski JM. 1993. Crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MgATP and peptide inhibitor. *Biochemistry 32*:2154–2161.